

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
22 March 2001 (22.03.2001)

PCT

(10) International Publication Number
WO 01/20011 A1

(51) International Patent Classification⁷: C12N 15/82,
9/02, 9/10, A01H 5/00, C12P 23/00 // (C12N 9/02, C12R
1:89)

(74) Agents: NILSSON, Brita et al.; AB Stockholms Patent-
byrå, Zacco & Bruhn, Box 23101, S-104 35 Stockholm
(SE).

(21) International Application Number: PCT/SE00/01767

(22) International Filing Date:
13 September 2000 (13.09.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
9903336-7 17 September 1999 (17.09.1999) SE

(71) Applicant (for all designated States except US): ASTAC-
AROTENE AB [SE/SE]; Idrottsvägen 4, S-134 40 Gus-
tavsberg (SE).

(72) Inventors; and

(75) Inventors/Applicants (for US only): HÖGLUND, Anna-
Stina [SE/SE]; Svanckärsvägen 27, S-756 53 Uppsala (SE).
STÅLBERG, Kjell [SE/SE]; Vänortsgatan 118, S-752 63
Uppsala (SE).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ,
DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR,
HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ,
NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM,
TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian
patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European
patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE,
IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG,
CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

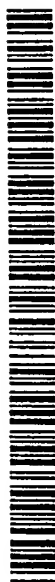
Published:

— With international search report.

For two-letter codes and other abbreviations, refer to the "Guid-
ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.

(54) Title: DNA CONSTRUCT AND ITS USE

(57) Abstract: A DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region is disclosed. The DNA construct may additionally comprise a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant. The peptide with enzyme activity is preferably a peptide with β -carotene C-4-oxygenase activity, e.g. from the alga *Chlorella zofingiensis*. Comprised by the invention are also a transgenic oilseed plant cell, e.g. of rape, sunflower, soybean or mustard origin, and a transgenic oilseed plant-produced xanthophyll, such as canthaxanthin or astaxanthin, and also astaxanthin esters.



WO 01/20011 A1

DNA construct and its use.

The present invention relates to a new DNA construct for transformation into oilseed plants. The DNA construct comprises nucleotide sequences encoding peptides with enzyme activities necessary for the high-level production and esterification of keto group-containing xanthophylls in oilseed plants.

Background of the invention

Carotenoids are produced *de novo* by plants, fungi, algae and some bacteria. A number of biosynthetic steps are needed for the biological production of the carotenoids. There are two chemically different groups of carotenoids, namely carotenes containing only carbon and hydrogen molecules and xanthophylls containing oxygen in the molecule in addition to carbon and hydrogen.

The xanthophylls, and particularly astaxanthin (3,3'-dihydroxy- β - β -carotene-4,4'-dione), are often colored pigments and are used as such or as anti-oxidants.

Carotenes are biological precursors for the production of the oxygen-containing xanthophylls. There are two types of enzymes responsible for the introduction of hydroxy groups and keto groups into the carotenes, namely hydroxylases and ketolases, respectively.

The keto group-containing xanthophyll astaxanthin, which has keto and hydroxy groups, is biosynthetically produced from beta-carotene.

Large-scale production of xanthophylls from natural sources is at present performed by AstaCarotene AB, Gustavsberg, Sweden, by cultivation of the alga *Haematococcus pluvialis* for the production of astaxanthin in esterified form.

It would be desirable to be able to produce keto group-containing xanthophylls particularly astaxanthin, in oilseed plants. Oilseed plants have naturally β -carotene hydroxylases but lack β -carotene C-4-oxygenase enzymes or ketolases.

Description of the invention

The present invention provides DNA constructs enabling and promoting production of keto group containing xanthophylls, especially astaxanthin, in oilseed plants, such as rape, sunflower, soybean and mustard. The DNA construct is transformed into the oilseed plant cell for expression of a protein or fused protein which has an enzyme activity enabling keto group insertion into a carotene or hydroxy carotene for the biosynthetic production of a keto group containing xanthophyll, such as cantaxanthin (β , β -carotene-4,4'-dione) and/or astaxanthin. Use is thus made of the biosynthetic pathway of the oilseed plant to

produce carotenoids. The naturally occurring synthesis of carotenoids involves a number of enzymes, namely 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and β -carotene C-4-oxygenase. Genes coding for peptides having these enzymatic activities may be inserted into the DNA construct of the invention, one or several per construct, to promote high-level production in the transgenic oilseed plant. In case only one enzyme coding gene is inserted per plant, two or more plants may be sexually interbred to produce plants containing all the desired enzyme activities.

Thus, the present invention is directed to a DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.

In a preferred embodiment of the invention the DNA construct additionally comprises between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity a nucleotide sequence coding for a transit peptide directing the translated fusion polypeptide to the chloroplast of the oilseed plant.

The DNA construct is preferably such that the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production is selected from the group consisting of peptides with 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, and β -carotene C-4-oxygenase activity. To promote esterification of astaxanthin a nucleotide sequence coding for a peptide with acyl transferase activity may be included in the group.

In a preferred embodiment of the DNA construct according to the invention the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence coding for a N-terminally truncated β -carotene C-4-oxygenase gene from the alga

Haematococcus pluvialis.

An example of the DNA construct of the invention is presented in the sequence listing as SEQ ID NO:1 and in Fig.1.

The present invention is also directed to a transgenic oilseed plant cell comprising the DNA construct of the invention, and preferably the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

The invention is additionally directed to transgenic oilseed plant-produced
5 xanthophyll, e.g. canthaxanthin and astaxanthin.

A preferred aspect of the invention is directed to transgenic oilseed plant-produced astaxanthin esters.

The present invention will now be illustrated with reference to the DNA construct disclosed in the sequence listing and in Fig.1, and the following description of
10 embodiments. However, the invention is not limited to these exemplifications.

Short description of the drawings

Fig.1 illustrates the nucleotide sequence of the DNA construct comprising the napin promoter, the chloroplast localization signal, the N-terminally truncated β -carotene C-4-oxygenase gene and the termination sequence, and the deduced amino acid sequences of the transit peptide
15 and the β -carotene C-4-oxygenase.

Description of embodiments

The invention is illustrated by production of astaxanthin in the seed of oilseed rape. The astaxanthin produced in the seed of the transgenic plant is extracted as part of the extracted oil. By use of conventionally used protocols for *Agrobacterium tumefaciens*
20 mediated transformation such as described by (Hoekema et al.1983, An et al. 1986, Fry et al. 1987, DeBlock et al. 1988, Radke et al.1988, or Moloney et al. 1989) transgenic plants are produced having a chimeric DNA construct that is genetically inherited and is able to produce astaxanthin. The nucleotide sequence of the chimeric DNA construct consist of four parts of different genetic origin namely: (1) a promoter, (2) a localization signal, (3) a β -carotene C-4-oxygenase coding region and (4) a termination sequence.
25

The napin promoter directs transcription to the seed of oilseed rape (Stålberg et al 1996). This promoter was coupled to a localization signal similar but not identical to a transit peptide (TP) of Rbcs1a (Krebbers, 1988) that directs the translated product of a fused gene to the chloroplast. The promoter and the TP sequence were ligated to a part of the coding
30 sequence of a ketolase gene BCK (Kajiwara et al. 1995). This enzyme oxygenates β -carotene to canthaxanthin, (Fraser et al. 1997). The chimeric DNA construct was then coupled to a suitable termination sequence, e.g. that of the *Agrobacterium tumefaciens* nopaline synthase gene (the nos 3' end)(Bevan et al. 1983), as illustrated in Fig.1.

Cellular storage of Astaxanthin

The storage of large amounts of free astaxanthin in plants will be difficult due to toxic effects of the molecule as it intercalates in the plant membranes. An effective esterification of astaxanthin to fatty acids enables storage of the esterified molecules in triacylglycerol containing oleosomes. Thus, an acyl transferase can be claimed to be of fundamental importance for the process, as is proteins that can mediate transport of different forms of astaxanthin from the chloroplast to the vesicles.

Sequences and oligonucleotides used in the construction of the DNA construct*1. Napin promoter (GeneBank ACCESSION No. J02798)*

This promoter sequence, a 1145 base pair fragment including the 5' leader sequence has a unique HindIII site at the 5' end. The 3' end was synthesized with an additionally 6 nucleotide BamHI site.

2. Transit peptide similar to RBCS1a (GeneBank ACCESSION No. X13611, X14565)

The transit peptide (TP) was amplified by PCR from -28 to the end of the transit cleavage aa=54/55 site of the Rbcs1a gene. The 5' end was synthesized with a BamHI site and similarly the 3' sequence was synthesized with a XbaI site. The two following oligonucleotides were used for the PCR amplification.

BamHI

5' primer: TP1 5'AGAC GGATCC TCAGTCACACAAAGAGTA 3'

SacI XbaI

3' primer: TP2 5'GTTC GAGCTC TCTAGA CATGCAGTTAACGC 3'

3. BCK (β -carotene C-4 oxygenase) (Genebank ACCESSION No. D45881)

The BCK fragment was amplified by PCR including a 5' XbaI site and was ligated to the TP already described. The 5' primer (BCK1) used for PCR, is homologous to the BCK sequence from nucleotide 264 and the 3' oligonucleotide (Ax40) ends with a stop codon and was synthesized with a SacI restriction site for cloning. The synthesized fragment was fused to the TP as shown in Fig 1.

Oligonucleotides used for PCR:

XbaI

5' primer: BCK1 5'ACAG TCTAGA ATGCCATCCGAGTCGTCA 3'

SacI

3' primer: AX40 5'CACCGAGCTCCATGACACTCTTGTGCAGA 3'

Description of SEQ ID NO:1 and SEQ ID NO:2

The sequences shown in Fig.1 are the same as the two sequences which are shown in the sequence listing.

The SEQ ID NO:1 is a nucleotide sequence composed of the following features:

5		Nucleotide No.
	Cloning site HindIII	1-6
	Napin Promoter	1-1145
	Cloning site BamHI	1146-1151
	Transit peptide leader	1152-1178
10	Transit peptide coding	1179-1347
	Cloning site XbaI	1348-1353
	β -carotene C-4-oxygenase	1354-2217
	β -carotene C-4-oxygenase 3' untranslated	2218-2266
	Cloning site SacI	2267-2272
15	Nopaline synthetase termination	2273-2536
	Cloning site EcoRI	2538-2543

The SEQ ID NO: 2 is a deduced amino acid sequence of the fusion protein of the transit peptide and the peptide with β -carotene C-4-oxygenase activity.

References

- An G, Watson BD, Chiang CC (1986), Transformation of tobacco, tomato, potato and
5 Arabidopsis-thaliana using a binary vector system. Plant Physiology 81 (1) 301-305.
- Bevan M, Barnes WM and Chilton MD (1983). Structure and transcription of the nopaline
synthase gene region of T-DNA. Nucleic Acids Res. 11 (2), 369-385 .
- 10 DeBlock M, DeBrouwer D, Tenning P (1989). Transformation of Brassica napus and Brassica
oleracea using Agrobacterium tumefaciens and the expression of the BAR and NEO genes in
transgenic plants Plant Physiology 91:2, 694-701.
- Fraser PD, Miura Y, Misawa N, (1997). In vitro characterization of astaxanthin biosynthetic
15 enzymes. J Biol Chem. Mar 7;272(10):6128-35.
- Fry J, Barnason A, and Horsch RB, (1987). Transformation of Brassica napus with
Agrobacterium tumefaciens based vectors. Plant Cell Reports 6:321-325.
- 20 Hoekema A, Hirsch PR, Hooykas PJJ Schilperoort, (1983). A binary vector strategy based on
separation of vir and T-region of the Agrobacterium tumefaciens Ti-plasmid. Nature vol 303,
179-180.
- Josefsson LG, Lenman M, Ericson ML and Rask L, (1987). Structure of a gene encoding the
25 1.7 S storage protein, napin, from Brassica napus. J. Biol. Chem. 262 (25), 12196-12201.
- Kajiwara S, Kakizono T, Saito T, Kondo K, Ohtani T, Nishio N, Nagai S and Misawa N.
(1995). Isolation and functional identification of a novel cDNA for astaxanthin biosynthesis
from Haematococcus pluvialis, and astaxanthin synthesis in Escherichia coli Plant Mol. Biol.
30 29 (2), 343-352.

Krebbers E, Seurinck J, Herdies L, Cashmore AR and Timko MP, (1988). Four genes in two diverged subfamilies encode the rubulose-1, 5-bisphosphate carboxylase small subunit polypeptides of *Arabidopsis thaliana* Plant Mol. Biol. 11, 745-759.

- 5 Moloney M, Walker JM and Sharma KK, (1989). High efficiency transformation of *Brassica napus* using *Agrobacterium* vectors. Plant Cell Reports 8:238-242.

- Radke SE, Andrews BM, Moloney MM, Crouch ML, Kridl JC, Knauf VC (1988),
Transformation of *Brassica napus* using *Agrobacterium tumefaciens* – Developmentally
10 regulated Expression of a reintroduced napin gene. TAG, 75: (5) 685-694 .

Pua E-C, Mehra-Palta A, Nagy F and Chua N-H, (1987). Transgenic plants of *Brassica napus*. Biotechnology vol 5, 815-817.

- 15 Stålberg K, Ellerstöm M, Ezcurra I, Ablov S, Rask L (1996). Disruption of an overlapping E-box/ABRE motif abolished high transcription of the napA storage-protein promoter in transgenic *Brassica napus* seeds. Planta 199(4):515-9.

Claims

1. A DNA construct comprising in the 5' to 3' direction of transcription operably linked a promoter region directing transcription to the seed of an oilseed plant, a nucleotide sequence coding for at least one peptide with enzyme activity necessary for keto group
5 containing xanthophyll production and esterification in an oilseed plant and a transcriptional termination region.

2. The DNA construct according to claim 1, which between the promoter region and the nucleotide sequence coding for at least one peptide with enzyme activity additionally comprises a nucleotide sequence coding for a transit peptide directing the translated fusion
10 polypeptide to the chloroplast of the oilseed plant.

3. The DNA construct according to claim 1 or 2, wherein the promoter is a napin promoter, the peptide with enzyme activity necessary for keto group containing xanthophyll production and esterification is selected from the group consisting of peptides with, 1-D-deoxyxylulose 5-phosphate synthase, isopentenyl pyrophosphate:dimethylallyl pyrophosphate
15 isomerase, geranylgeranyl pyrophosphate synthase, phytoene synthase, phytoene desaturase, zeta-carotene desaturase, lycopene beta-cyclase, β -carotene hydroxylase, β -carotene C-4-oxygenase, and acyl transferase activity.

4. The DNA construct according to any one of claims 1 - 3, wherein the nucleotide sequence coding for a peptide with enzyme activity is a nucleotide sequence
20 coding for a N-terminally truncated β -carotene C-4-oxygenase gene from the alga *Haematococcus pluvialis*.

5. The DNA construct according to claim 4, wherein the nucleotide sequence is SEQ ID NO:1.

6. Transgenic oilseed plant cell comprising the DNA construct of any one of
25 claims 1-5 .

7. Transgenic oilseed plant cell according to claim 6, wherein the oilseed plant is selected from the group consisting of rape, sunflower, soybean and mustard.

8. Transgenic oilseed plant-produced xanthophyll.

9. Transgenic oilseed plant-produced xanthophyll according to claim 8, wherein
30 the xanthophyll is canthaxanthin

10. Transgenic oilseed plant-produced xanthophyll according to claim 8, wherein the xanthophyll is astaxanthin.

11. Transgenic oilseed plant-produced xanthophyll according to claim 8, wherein the xanthophyll is astaxanthin esters.

1/3

Napin promoter

AAGCTTTCTTCATCGGTGATTGATTCCCTTTAAAGACTTATGTTTCTTATCTTGCTTCTGA
GGCAAGTATTCAGTTACCAGTTACCACTTATATTCTGGACTTTCTGACTGCATCCTCATT
TTTCCAACATTTTAAATTTCACTATTGGCTGAATGCTTCTTCTTTGAGGAAGAAACAATT
CAGATGGCAGAAATGTATCAACCAATGCATATATACAAATGTACCTCTTGTTCTCAAAAC
ATCTATCGGATGGTTCCATTTGCTTTGTCATCCAATTAGTGACTACTTTATATTATTAC
TCCTCTTTATTACTATTTTCATGCGAGGTTGCCATGTACATTATATTTGTAAGGATTGAC
GCTATTGAGCGTTTTTCTTCAATTTTCTTTATTTTAGACATGGGTATGAAATGTGTGTTA
GAGTTGGGTTGAATGAGATATACGTTCAAGTGAAGTGGCATAACGTTCTCGAGTAAGGAT
GACCTACCCATTCTTGAGACAAATGTTACATTTTAGTATCAGAGTAAATGTGTACCTAT
AACTCAAATTCGATTGACATGTATCCATTCAACATAAAATTAAACCAGCCTGCACCTGCA
TCCACATTTCAAGTATTTTCAAACCGTTCGGCTCCTATCCACCGGGTGTAACAAGACGGA
TTCCGAATTTGGAAGATTTTGACTCAAATTCCTCAATTTATATTGACCGTGACTAAATCAA
CTTTAACTTCTATAATTCTGATTAAGCTCCCAATTTATATTCCCAACGGGCACTACCTCCA
AAATTTATAGACTCTCATCCCCTTTTAAACCAACTTAGTAAACGTTTTTTTTTTTAAATTT
TATGAAGTTAAGTTTTTACCTTGTTTTTAAAAAGAATCGTTCATAAGATGCCATGCCAGA
ACATTAGCTACACGTTACACATAGCATGCAGCCGCGGAGAATTGTTTTTCTTCGCCACTT
GTCACTCCCTTCAAACACCTAAGAGCTTCTCTCTCACAGCACACACATACAATCACATGC
GTGCATGCATTATTACACGTGATCGCCATGCAAATCTCCTTTATAGCCTATAAATTA
ACTCATCCGCTTCACTCTTTACTCAAACCAAACTCATCAATACAAACAAGATTAAAAACATA

End -28 untranslated leader TP start
CACGAGGATCCTCAGTCACACAAAGAGTAAAGAAGAACAATGGCTTCCTCTATGCTCTCT
M A S S M L S

TCCGCTACTATGGTTGCCTCTCCGGCTCAGGCCACTATGGTCGCTCCTTTCAACGGACTT
S A T M V A S P A Q A T M V A P F N G L

AAGTCCTCCGCTGCCTTCCCAGCCACCCGCAAGGCTAACAACGACATTACTTCCATCACA
K S S A A F P A T R K A N N D I T S I T

FIG. 1

2/3

TP End C-4-Oxygenase

AGCAACGGCGGACGCGTTAACTGCATGTCTAGAATGCCATCCGAGTCGTCAGACGCAGCT
S N G G R V N C M S R M P S E S S D A A

CGTCCTGCGCTAAAGCACGCCTACAAACCTCCAGCATCTGACGCCAAGGGCATCACGATG
R P A L K H A Y K P P A S D A K G I T M

GCGCTGACCATCATTGGCACCTGGACCGCAGTGTTTTTACACGCAATATTTCAAATCAGG
A L T I I G T W T A V F L H A I F Q I R

CTACCGACATCCATGGACCAGCTTCACTGGTTGCCTGTGTCCGAAGCCACAGCCCAGCTT
L P T S M D Q L H W L P V S E A T A Q L

TTGGGCGGAAGCAGCAGCCTACTGCACATCGCTGCAGTCTTCATTGTACTTGAGTTCCTG
L G G S S S L L H I A A V F I V L E F L

TACACTGGTCTATTCATCACCACACATGACGCAATGCATGGCACCATAGCTTTGAGGCAC
Y T G L F I T T H D A M H G T I A L R H

AGGCAGCTCAATGATCTCCTTGGCAACATCTGCATATCACTGTACGCCTGGTTTGACTAC
R Q L N D L L G N I C I S L Y A W F D Y

AGCATGCTGCATCGCAAGCACTGGGAGCACCACAACCATACTGGCGAAGTGGGGAAAGAC
S M L H R K H W E H H N H T G E V G K D

CCTGACTTCCACAAGGGAAATCCCGGCCTTGTCCCCTGGTTCGCCAGCTTCATGTCCAGC
P D F H K G N P G L V P W F A S F M S S

TACATGTCCCTGTGGCAGTTTGCCCGGCTGGCATGGTGGGCAGTGGTGATGCAAATGCTG
Y M S L W Q F A R L A W W A V V M Q M L

GGGGCGCCCATGGCAAATCTCCTAGTCTTCATGGCTGCAGCCCCAATCTTGTGAGCATTC
G A P M A N L L V F M A A A P I L S A F

CGCCTCTTCTACTTCGGCACTTACCTGCCACACAAGCCTGAGCCAGGCCCTGCAGCAGGC
R L F Y F G T Y L P H K P E P G P A A G

TCTCAGGTGATGGCCTGGTTCAGGGCCAAGACAAGTGAGGCATCTGATGTGATGAGTTTC
S Q V M A W F R A K T S E A S D V M S F

CTGACATGCTACCACTTTGACCTGCACTGGGAGCACCACAGATGGCCCTTTGCCCCCTGG
L T C Y H F D L H W E H H R W P F A P W

C-4 oxygenase Stop

TGGCAGCTGCCCCACTGCCGCGCCTGTCCGGGCGTGGCCTGGTGCCTGCCTTGGCATGA
W Q L P H C R R L S G R G L V P A L A *

FIG.1 (cont.)

3/3 .

[illegible]

Fig.1 (cont.)

SEQUENCE LISTING

<110> AstaCarotene AB

<120> DNA construct and its use

<130> 29295-AstaCarotene

<140>

<141>

<160> 2

<170> PatentIn Ver. 2.1

<210> 1

<211> 2543

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: napin promoter
+ chloroplast localization signal + beta-carotene C-4 oxygenase
coding sequence + termination sequence

<220>

<221> promoter

<222> (1)..(1145)

<220>

<221> transit_peptide

<222> (1179)..(1347)

<220>

<221> CDS

<222> (1179)..(2217)

<220>

<221> terminator

<222> (2273)..(2536)

<400> 1

aagctttctt catcggtgat tgattccttt aaagacttat gtttcttata ttgctttctga 60

ggcaagtatt cagttaccag ttaccactta tattctggac tttctgactg catcctcatt 120

tttccaacat tttaaatttc actattggct gaatgcttct tctttgagga agaaacaatt 180

cagatggcag aaatgtatca accaatgcat atatacaaat gtacctcttg ttctcaaaac 240

atctatcgga tgggtccatt tgctttgtca tccaattagt gactacttta tattattcac 300

tcctctttat tactattttc atgcgaggtt gccatgtaca ttatatttgt aaggattgac 360

gctattgagc gtttttcttc aattttcttt attttagaca tgggtatgaa atgtgtgtta 420

gagttgggtt gaatgagata tacgttcaag tgaagtggca taccgttctc gagtaaggat 480

gacctacca ttcttgagac aaatgttaca ttttagtatc agagtaaaat gtgtacctat 540

aactcaaatt	cgattgacat	gtatccattc	aacataaaat	taaaccagcc	tgcacctgca	600
tccacatttc	aagtattttc	aaaccgttcg	gctcctatcc	accgggtgta	acaagacgga	660
ttccgaat	ttt ggaagatt	ttt gactcaa	att cccaatt	tat attgacc	gtg actaaat	caa 720
ctttaacttc	tataattctg	attaagctcc	caatttatat	tcccaacggc	actacctcca	780
aaatttatag	actctcatcc	ccttttaaac	caacttagta	aacgtttttt	tttttaattt	840
tatgaagtta	agtttttacc	ttgtttttta	aaagaatcgt	tcataagatg	ccatgccaga	900
acattagcta	cacgttacac	atagcatgca	gccgcggaga	attgtttttc	ttcgccactt	960
gtcactccct	tcaaacacct	aagagcttct	ctctcacagc	acacacatac	aatcacatgc	1020
gtgcatgcat	tattacacgt	gatcgccatg	caaatctcct	ttatagccta	taaattaact	1080
catccgcttc	actctttact	caaaccaaaa	ctcatcaata	caaacaagat	taaaaacata	1140
cacgaggatc	ctcagtcaca	caaagagtaa	agaagaaca	atg gct tcc tct atg		1194
				Met Ala Ser Ser Met		
				1	5	
ctc tct tcc gct act atg gtt gcc tct ccg gct cag gcc act atg gtc						1242
Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala Gln Ala Thr Met Val						
	10		15		20	
gct cct ttc aac gga ctt aag tcc tcc gct gcc ttc cca gcc acc cgc						1290
Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala Phe Pro Ala Thr Arg						
	25		30		35	
aag gct aac aac gac att act tcc atc aca agc aac ggc gga cgc gtt						1338
Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser Asn Gly Gly Arg Val						
	40		45		50	
aac tgc atg tct aga atg cca tcc gag tcg tca gac gca gct cgt cct						1386
Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser Asp Ala Ala Arg Pro						
	55		60		65	
gcg cta aag cac gcc tac aaa cct cca gca tct gac gcc aag ggc atc						1434
Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser Asp Ala Lys Gly Ile						
	70		75		80	85
acg atg gcg ctg acc atc att ggc acc tgg acc gca gtg ttt tta cac						1482
Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr Ala Val Phe Leu His						
	90		95		100	
gca ata ttt caa atc agg cta ccg aca tcc atg gac cag ctt cac tgg						1530
Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met Asp Gln Leu His Trp						
	105		110		115	
ttg cct gtg tcc gaa gcc aca gcc cag ctt ttg ggc gga agc agc agc						1578
Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu Gly Gly Ser Ser Ser						
	120		125		130	
cta ctg cac atc gct gca gtc ttc att gta ctt gag ttc ctg tac act						1626
Leu Leu His Ile Ala Ala Val Phe Ile Val Leu Glu Phe Leu Tyr Thr						
	135		140		145	

ggt cta ttc atc acc aca cat gac gca atg cat ggc acc ata gct ttg 1674
 Gly Leu Phe Ile Thr Thr His Asp Ala Met His Gly Thr Ile Ala Leu
 150 155 160 165

agg cac agg cag ctc aat gat ctc ctt ggc aac atc tgc ata tca ctg 1722
 Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn Ile Cys Ile Ser Leu
 170 175 180

tac gcc tgg ttt gac tac agc atg ctg cat cgc aag cac tgg gag cac 1770
 Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg Lys His Trp Glu His
 185 190 195

cac aac cat act ggc gaa gtg ggg aaa gac cct gac ttc cac aag gga 1818
 His Asn His Thr Gly Glu Val Gly Lys Asp Pro Asp Phe His Lys Gly
 200 205 210

aat ccc ggc ctt gtc ccc tgg ttc gcc agc ttc atg tcc agc tac atg 1866
 Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe Met Ser Ser Tyr Met
 215 220 225

tcc ctg tgg cag ttt gcc cgg ctg gca tgg tgg gca gtg gtg atg caa 1914
 Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp Ala Val Val Met Gln
 230 235 240 245

atg ctg ggg gcg ccc atg gca aat ctc cta gtc ttc atg gct gca gcc 1962
 Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val Phe Met Ala Ala Ala
 250 255 260

cca atc ttg tca gca ttc cgc ctc ttc tac ttc ggc act tac ctg cca 2010
 Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe Gly Thr Tyr Leu Pro
 265 270 275

cac aag cct gag cca ggc cct gca gca ggc tct cag gtg atg gcc tgg 2058
 His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser Gln Val Met Ala Trp
 280 285 290

ttc agg gcc aag aca agt gag gca tct gat gtg atg agt ttc ctg aca 2106
 Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val Met Ser Phe Leu Thr
 295 300 305

tgc tac cac ttt gac ctg cac tgg gag cac cac aga tgg ccc ttt gcc 2154
 Cys Tyr His Phe Asp Leu His Trp Glu His His Arg Trp Pro Phe Ala
 310 315 320 325

ccc tgg tgg cag ctg ccc cac tgc cgc cgc ctg tcc ggg cgt ggc ctg 2202
 Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu Ser Gly Arg Gly Leu
 330 335 340

gtg cct gcc ttg gca tgacctgggc cctccgctgg tgaccagcg tctgcacaag 2257
 Val Pro Ala Leu Ala
 345

agtgtcatgg agctcgaatt tccccgatcg ttcaaacatt tggcaataaa gtttcttaag 2317

attgaatcct gttgccgggc ttgcgatgat tatcatataa tttctgttga attacgttaa 2377

gcatgtaata attaacaatgt aatgcatgac gttatattatg agatggggttt ttatgattag 2437

agtccccgcaa ttatacattt aatacgcgat agaaaacaaa atatagcgcg caaactagga 2497

taaattatcg cgcgcgggtgt catctatggt actagatcgg gaattc 2543

<210> 2

<211> 346

<212> PRT

<213> Artificial Sequence

<223> Description of Artificial Sequence: deduced fusion protein of
transit peptide + peptide with beta-carotene C-4 oxygenase activity

<400> 2

Met Ala Ser Ser Met Leu Ser Ser Ala Thr Met Val Ala Ser Pro Ala

1

5

10

15

Gln Ala Thr Met Val Ala Pro Phe Asn Gly Leu Lys Ser Ser Ala Ala

20

25

30

Phe Pro Ala Thr Arg Lys Ala Asn Asn Asp Ile Thr Ser Ile Thr Ser

35

40

45

Asn Gly Gly Arg Val Asn Cys Met Ser Arg Met Pro Ser Glu Ser Ser

50

55

60

Asp Ala Ala Arg Pro Ala Leu Lys His Ala Tyr Lys Pro Pro Ala Ser

65

70

75

80

Asp Ala Lys Gly Ile Thr Met Ala Leu Thr Ile Ile Gly Thr Trp Thr

85

90

95

Ala Val Phe Leu His Ala Ile Phe Gln Ile Arg Leu Pro Thr Ser Met

100

105

110

Asp Gln Leu His Trp Leu Pro Val Ser Glu Ala Thr Ala Gln Leu Leu

115

120

125

Gly Gly Ser Ser Ser Leu Leu His Ile Ala Ala Val Phe Ile Val Leu

130

135

140

Glu Phe Leu Tyr Thr Gly Leu Phe Ile Thr Thr His Asp Ala Met His

145

150

155

160

Gly Thr Ile Ala Leu Arg His Arg Gln Leu Asn Asp Leu Leu Gly Asn

165

170

175

Ile Cys Ile Ser Leu Tyr Ala Trp Phe Asp Tyr Ser Met Leu His Arg

180

185

190

Lys His Trp Glu His His Asn His Thr Gly Glu Val Gly Lys Asp Pro

195

200

205

Asp Phe His Lys Gly Asn Pro Gly Leu Val Pro Trp Phe Ala Ser Phe

210

215

220

Met Ser Ser Tyr Met Ser Leu Trp Gln Phe Ala Arg Leu Ala Trp Trp

225

230

235

240

Ala Val Val Met Gln Met Leu Gly Ala Pro Met Ala Asn Leu Leu Val

245

250

255

Phe Met Ala Ala Ala Pro Ile Leu Ser Ala Phe Arg Leu Phe Tyr Phe
260 265 270

Gly Thr Tyr Leu Pro His Lys Pro Glu Pro Gly Pro Ala Ala Gly Ser
275 280 285

Gln Val Met Ala Trp Phe Arg Ala Lys Thr Ser Glu Ala Ser Asp Val
290 295 300

Met Ser Phe Leu Thr Cys Tyr His Phe Asp Leu His Trp Glu His His
305 310 315 320

Arg Trp Pro Phe Ala Pro Trp Trp Gln Leu Pro His Cys Arg Arg Leu
325 330 335

Ser Gly Arg Gly Leu Val Pro Ala Leu Ala
340 345

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/01767

A. CLASSIFICATION OF SUBJECT MATTER

IPC7: C12N 15/82, C12N 9/02, C12N 9/10, A01H 5/00, C12P 23/00 // (C12N 9/02, C12R 1:89)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC7: C12N, C12P, A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9907867 A1 (CALGENE LLC), 18 February 1999 (18.02.99), see abstract, page 13, lines 15-23, claims --	1-11
X	WO 9806862 A1 (CALGENE, INC.), 19 February 1998 (19.02.98), see page 8. line 9 - page 12, line 15; page 13, line 22 - page 15, line 5 --	1-11
X	Susan Budavari et al "THE MERCK INDEX", twelfth edition", 1996, MERCK & CO., INC. NJ, see entries 890, "Astaxanthin"; 1798, "Canthaxanthin"; 10197, "Xanthophyll". --	8-10

☒ Further documents are listed in the continuation of Box C.☒ See patent family annex.

* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

12 December 2000

Date of mailing of the international search report

- 20 -12- 2000

Name and mailing address of the ISA/
Swedish Patent Office
Box 5055, S-102 42 STOCKHOLM
Facsimile No. +46 8 666 02 86

Authorized officer

Hampus Rystedt/GH
Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/01767

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 9818910 A1 (YISSUM RESEARCH AND DEVELOPMENT COMPANY OF THE HEBREW UNIVERSITY OF JERUSALEM), 7 May 1998 (07.05.98), see abstract, page 28, line 24 - page 29, line 4	1-4
A	--	5-11
A	WO 9613149 A1 (AMOCO CORPORATION), 9 May 1996 (09.05.96)	1-11
A	--	
A	EMBL/GenBank/DDBJ databases, accession no. X86782, 1997-11-30, Harker M. et al: "H.pluvialis mRNA for beta-carotene C-4 oxygenase"	4,5
A	--	
A	EMBL/GenBank/DDBJ databases, accession no. D45881, 1995-12-29, Kajiwarea S.: "Haematococcus pluvialis mRNA for bet-carotene ketolase, complete cds"	3
A	--	
A	EMBL/GenBank/DDBJ databases, accession no. X86783, 1998-06-02, Harker M. et al: "H.pluvialis mRNA for phyteone desaturase"	3
A	--	
A	EMBL/GenBank/DDBJ databases, accession no. AF082325, Sun Z. et al: "Haematococcus pluvialis isopentenyl pyrophosphate:dimethylallyl pyrophosphate isomerase (ipiHp1) mRNA, complete cd, 1998-08-18	3
X	--	
X	EMBL/GenBank/DDBJ databases, accession no. AF082326, 1998-08-18, Sun Z. et al: "Haematococcus pluvialis isopenetyl pyrophosphate:dimethylallyl pyrophosphate isomerase (ipiHp2) mRNA, complete cds"	3
	--	

INTERNATIONAL SEARCH REPORT

International application No.

PCT/SE 00/01767

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EMBL/GenBank/DDBJ databases, accession no. AF162276, 1999-09-10, Linden H.: "Haematococcus pluvialis carotenoid hydroxylase mRNA, partial cds" --	3
A	WO 9930701 A1 (ASTACAROTENE), 24 June 1999 (24.06.99), see abstract and claims --	11
A	WO 9837874 A1 (ASTACAROTENE AB), 3 Sept 1998 (03.09.98), see abstract and claims --	11
A	JOURNAL OF PHOTOCHEMISTRY AND PHOTOBIOLOGY B, Volume 30, 1995, BISWAL, B et al, "Carotenoid catabolism durint leaf senescence and its control by light" page 3 - page 13 -- -----	11

INTERNATIONAL SEARCH REPORT

International application No.
SE00/01767

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see extra sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

According to Article 34 (3) (a-c) and Rule 13.2, an international application shall relate to one invention only or to a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art. The present application relates to five such groups of inventions, namely:

1. A DNA construct encoding an enzyme in the carotenoid biosynthetic pathway and cells expressing the enzyme, according to claims 1-7.
2. Transgenic oilseed plant-produced xanthophyll, according to claim 8.
3. Transgenic oilseed plant-produced canthaxanthin, according to claim 9.
4. Transgenic oilseed plant-produced astaxanthin, according to claim 10.
5. Transgenic oilseed plant-produced astaxanthin esters, according to claim 11.

The feature common to all inventions is the transgenic production of carotenoids in oilseed plants. However, this feature is already known through WO-A1-9806862. The production of different carotenoids, and DNA constructs facilitating the production, is thus not linked by a special technical feature as required by Rule 13.2. As the additional effort of searching inventions 2-5 does not justify an additional search fee, all inventions have been searched.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.

PCT/SE 00/01767

Patent document cited in search report			Publication date	Patent family member(s)		Publication date
WO	9907867	A1	18/02/99	AU	8900298 A	01/03/99
				EP	1002117 A	24/05/00
WO	9806862	A1	19/02/98	AU	4058497 A	06/03/98
				BR	9713462 A	28/03/00
				CN	1227609 A	01/09/99
				EP	0925366 A	30/06/99
WO	9818910	A1	07/05/98	AU	4743697 A	22/05/98
				NO	991996 A	22/06/99
				US	5916791 A	29/06/99
				US	5965795 A	12/10/99
				CN	1247565 A	15/03/00
				EP	0951534 A	27/10/99
				PL	332965 A	25/10/99
WO	9613149	A1	09/05/96	AU	697358 B	01/10/98
				AU	3970195 A	23/05/96
				CA	2203815 A	09/05/96
				CN	1172416 A	04/02/98
				EP	0792352 A	03/09/97
				JP	10509309 T	14/09/98
				NO	971945 A	27/06/97
				NZ	296012 A	28/05/99
				PL	319788 A	01/09/97
				US	5618988 A	08/04/97
WO	9930701	A1	24/06/99	AU	1897299 A	05/07/99
				EP	1049460 A	08/11/00
				NO	20003042 A	14/06/00
				SE	511237 C	30/08/99
				SE	9704693 A	17/06/99
WO	9837874	A1	03/09/98	AU	719090 B	04/05/00
				AU	2796797 A	19/11/97
				AU	6295198 A	18/09/98
				CN	1248912 T	29/03/00
				EP	0898823 A	03/03/99
				EP	0981338 A	01/03/00
				NO	994109 A	27/10/99
				PL	335370 A	25/04/00
				SE	9700708 A	28/08/98

THIS PAGE BLANK (USPTO)